Structure, Formation, Mechanical Properties, and Disposal of the Embryo Attachment System of an Estuarine Crab, *Sesarma haematocheir*

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Abstract. In most decapod crustaceans, fertilized eggs extruded from the gonopore attach to ovigerous hairs within the incubation chamber of the female. The attachment is effected by an "embryo attachment system." The three continuous components of this system are the egg envelope, the funiculus, and the investment coat, which wraps around an ovigerous hair. Transmission electron microscopy (TEM) revealed that the embryo of *Sesarma haematocheir* is enfolded by three distinct envelopes (E1, E2, and E3), whereas the embryo attachment system is composed of only the outermost, single envelope (E1) with two sublayers (E1a and E1b). This envelope (E1) originates from the outer layer of the vitelline membrane (envelope of the ovum) with two sublayers (E1a′ and E1b′). The sequence and timing of events in the formation of the embryo attachment system was determined on the basis of observations of female behavior, ultrastructure, and mechanical properties of the membranes. The egg envelope (E1a′ + E1b′) is not adhesive immediately after extrusion from the gonopore; but 5 min after egg-laying, it becomes adhesive—a change associated with "fusion" of the two sublayers (E1)—and attaches the eggs to the ovigerous hairs from 5 to 30 min after egg-laying. The layer E1a′ always binds to an ovigerous hair at specific, electron-dense attachment sites that are distributed longitudinally on the surface of each hair. Plasticity of the egg envelope changes, and the female kneads her eggs by the movement of ovigerous setae; this movement forms the investment coat on the ovigerous hair (10–40 min after egg-laying). Thirty minutes after egg-laying, the egg envelope again divides into two sublayers (E1a and E1b), and the adhesiveness rapidly decreases. The plasticity of the envelope remains, and the funiculus is formed, accompanied by kneading of the eggs (40–90 min after egg-laying). The embryos hatch one month after incubation, and the attachment systems all slip off their ovigerous hairs by the actions of the ovigerous-hair slipping substance (OHSS). This substance appears to act specifically at the attachment sites on the hair, lysing the bond with layer E1a, and thereby disposing of the embryonic attachment system and preparing the hairs for the next clutch of embryos.

Introduction

The oocytes of some decapod crustaceans are fertilized when they pass through the seminal receptacles where sperms are stored (Hartnoll, 1968). Fertilization is followed by egg-laying; that is, the eggs are extruded through two gonopores on the ventral side of the thorax of the female. The number of eggs extruded at once varies with species and the duration of incubation; for example, an estuarine semi-terrestrial crab, *Sesarma haematocheir*, laid 10,000–50,000 eggs in one batch. After laying, the eggs attach to ovigerous hairs borne on the maternal ovigerous seta; the attachment is through a stalk called the funiculus (Herrick, 1895; Yonge, 1937, 1946, 1955; Cheung, 1966; Fisher and Clark, 1983; Goudeau and Lachaise, 1983). The mechanism by which the funiculus forms and attaches to the ovigerous hairs, as well as the process by which the embryonic envelope forms, have been subjects of controversy for many years.

Yonge (1937) reported that many secretory glands occur on the pleopods of the female of the lobster *Homarus*

vulgaris. These glands are identical in structure to the teg- umental glands that occur everywhere beneath the epithe- lium, and Yonge speculated that the fertilized eggs are attached by a "cement" secreted by these glands at the time of egg-laying. Observations by Andrews (1906), Stephens (1952), and Aiken and Waddy (1982) supported Yonge's hypothesis. Moreover, Fisher and Clark (1983) reported that the fertilized eggs of an estuarine shrimp, *Palaemon mac- rodactylus,* are attached to each other, or to the ovigerous hairs of the maternal pleopods, by materials that are pro- duced by the pleopods and form the outer layer of the embryonic envelope.

In contrast, a study of embryonic envelope formation and egg attachment in the shore crab, *Carcinus maenas,* indi- cated that the funiculus forms from the outermost layer of the "trichromatic membranes," all of which are produced by mature eggs after fertilization (Cheung, 1966). Cheung found no "cement glands" on the pleopod of this crab. Cheung's hypothesis was also supported by the ultrastruc- tural studies of Goudeau and coworkers, who showed that the funiculus of *Carcinus maenas* is an extension of the two vitelline layers of the mature egg. These two layers are altered structurally and stretched by the vigorous beating of the maternal pleopods; by this action the material in the layers wraps around the ovigerous hairs without involving any adhesive substance (Goudeau and Lachaise, 1980, 1983; Goudeau *et al.,* 1987; reviewed by Hinsch, 1990).

The embryo attachment system of many intertidal and estuarine crabs includes, not only the embryonic envelope and the funiculus, but also a clear "coat" that invests the whole hair (Saigusa, 1994). If the newly extruded eggs are attached to the ovigerous hairs by a "cement" or similar substance that is secreted from the female, we should ob- serve two kinds of material on the ovigerous hair: that is, a layer secreted by the maternal pleopods, and another layer originating from the egg envelope. On the other hand, if the egg envelope has only been stretched and wrapped around the ovigerous hairs, the coat should consist only of layers originating from the egg envelope. To solve this problem, the modification of the egg envelope of *Sesarma haemato- cheir*—an estuarine terrestrial crab—was investigated mor- phologically, and physiological changes in the properties of the envelope are described in terms of adhesion and plas- ticity. On the basis of these results, we present a schedule for an egg attachment for this crab.

Another purpose of this study was to investigate the disassociation of the embryo attachment system from the ovigerous hair. When embryonic development is complete, hatching occurs in synchrony with the times of high tide (Saigusa, 1992, 1993; Saigusa and Terajima, 2000). An active factor that we call OHSS (ovigerous-hair stripping substance) is released by the embryos at the time of hatch- ing, causing the investment coat and the rest of the embryo attachment system to slip off the ovigerous hairs several hours after larval release. This process prepares the hairs for the attachment of the next clutch of the embryos (for further details, see Saigusa, 1994, 1995, 1996). But the mechanism by which the investment coat slips off the hairs is not yet known. OHSS might cause morphological changes to the coat. Thus, we have examined ultrastructural changes to investment coats that were treated with OHSS solution and were subsequently stripped off their hairs.

Materials and Methods

Animals and strategy for experiment

The animal used for this study was *Sesarma haemato- cheir* (red-handed crab; akate-gani) inhabiting thickets along estuaries in western Japan. The females of this species incubate two to three successive clutches of developing embryos between June and September. After one month of incubation, the embryos hatch, and the zoea larvae are released in the water (Saigusa, 1982). The number of fe- males releasing larvae increases at about the time of full and new moons and decreases at the half moons—a semilunar rhythm.

The most important procedure for this study was to collect females that had just begun to lay their eggs. Egg- laying occurs primarily in the morning, from 0600 to 1200, but it begins with no visual sign, so in the field we cannot specify precisely any particular female that has just started laying eggs. Therefore, these investigations were carried out in the laboratory. We maintained a few hundred females in the laboratory, which enhanced the chance that we could observe females that were starting egg-laying. We also took advantage of the fact that, in *S. haematocheir,* most females incubate their next clutch within a few days after a larval release. Therefore if females that had released zoea larvae during any night were placed in different containers, we could expect that many of them would start egg-laying again a few days later. Thus, this species was well suited for the present study.

Maintenance of crabs and observations of egg-laying

Females used in this study were collected at Kasaoka, Okayama prefecture, Japan (Saigusa, 1982). Ovigerous fe- males on the way to water would cross a road along the seacoast just after sunset, so each evening at this spot, 50–200 females were collected and transported to the lab- oratory. After the females released their larvae, they were washed repeatedly with a large quantity of tap water to remove adherent hatch water and any zoeas still attached. The females were then kept in plastic containers (70 cm long, 50 cm wide, and 30 cm in depth), each containing a small quantity of seawater diluted less than 5‰. Females that had released larvae each night were moved to different containers. The containers were exposed to the natural day-

night cycle in the laboratory (sunset at 1830–1920 and sunrise at 0450–0530 in July–August). Within a few days, most females began to incubate their new clutch of fertilized eggs. A board in the container, providing shelter for the crabs (see Saigusa, 1980), was gently removed at times, and any female that had just started egg-laying was very carefully isolated to a transparent vessel so that egg-laying and subsequent behaviors could be observed, and the eggs were retrieved.

Transmission electron microscopy

Fertilized eggs for microscopy were obtained from the abdomen after egg-laying (Fig. 1A and 1B), whereas mature, but unfertilized (unextruded) eggs were obtained directly from the ovary. These eggs and embryos were fixed for 1.5 h at room temperature in 2.5% glutaraldehyde dissolved in a cacodylate buffer (400 mM NaCl, 100 mM Na-cacodylate, pH 7.4), and then washed. These egg samples were further post-fixed for 1 h, at 4 °C, with 1% OsO₄ dissolved in the cacodylate buffer. After post-fixation, the specimens were dehydrated through a graded series of ethanol solutions and embedded in a low-viscosity epoxy resin (TAAB). Ultrathin sections were cut with a Porter-Blum MT2-B ultramicrotome and collected on copper grids. These sections were further stained with 1% uranyl acetate dissolved in 50% ethanol, and with 0.1% lead citrate, and were then examined with a Hitachi H500H electron microscope operated at 75 kV.

Assaying the adherence and plasticity of the egg envelope

With a fine forceps, 5–20 fertilized eggs were removed every 10 min from a female just after egg-laying (Fig. 1B). These eggs were placed in a polystyrene petri dish (Falcon 1007; Becton Dickinson Labware, NJ) with seawater diluted to one-third (10‰). Characteristically, when an egg adhered to the bottom of the petri dish it would not roll, even if the dish were slowly shaken by hand (Fig. 1C). The strength of envelope adherence was estimated as the percentage of eggs that adhered to the bottom of a dish. If these adherent eggs were gently rolled with a forceps, the envelope elongated (Fig. 1D). While one end of the elongated envelope (*a*) remained attached to the bottom of the dish, the opposite end (*a'*) still enclosed the egg. If the egg was rolled sufficiently, either the envelope or the egg would break. At this point, the length of the envelope (*a–a'*) was taken as a rough estimate of its plasticity. A new petri dish was used for each test of eggs removed from a female.

Action of OHSS on the embryo attachment system: fine structure

The embryo attachment system slips off of the ovigerous hair after hatching. This is due to an active factor released

from the embryo at the time of hatching. This factor, which we call OHSS (ovigerous-hair stripping substance), is contained in the hatch water of crabs (Saigusa, 1994, 1995, 1996). In this study, ovigerous seta with premature embryos attached were separated from a few ovigerous females and cut into three pieces each. The tip of the seta was discarded, and each cluster of embryos was placed in a well of a plastic culture dish and incubated with 0.5 ml of concentrated hatch water (see Saigusa and Iwasaki, 1999). Control embryo clusters were immersed in 0.5 ml of 20 mM Tris-HCl buffer (pH 8.5). Each segment of embryo cluster was removed from the hatch water, or the control buffer, after 2 min, 4 min, 6 min, 8 min, 10 min, 20 min, 60 min, and 120 min, and each was fixed for transmission electron microscopy. The fixation and ultrathin sectioning were carried out as described above.

Results

Ultrastructure of the embryo attachment system in the incubating female

Egg envelope. The female of *Sesarma haematocheir* has four pairs of abdominal appendages; each bears one plumose and one non-plumose ovigerous seta (see Saigusa, 1994). Embryos attach to the ovigerous hairs by a stalk, the funiculus, and rarely to each other (Fig. 2A). The funiculus never is attached directly to an ovigerous hair, but rather to a coat that invests the hair. Figure 2B shows the fine structure of the egg envelope. The envelope consists of three layers: the outermost (E1), the thick middle (E2), and the innermost thin (E3) layers. The outermost layer further consists of two distinguishable sublayers designated E1a and E1b.

Funiculus. Figure 3A shows the base of the funiculus at its junction with the embryo. The funiculus consists of a single layer, 0.3–0.4 μm thick, which is continuous with the outermost layer (E1) investing the embryo. Moreover, the two sublayers (E1a and E1b) are also present in the funiculus (Fig. 3B). In contrast, no component of E2 or E3 was ever found in the funiculus. The proximal end of the funiculus is folded onto and wrapped around the ovigerous hair to produce the stratified investment coat (Fig. 3C). The coat usually invests only one hair, but a few hairs are sometimes included in the wrapping. The thickness of the coat differs from one hair to the next; for example, in Figure 3C, the coat is only 2–3 μm at its thinnest, but 25 μm at its thickest. In every part, the investment coat, like the funiculus, includes only the E1 layer and its components (E1a and E1b). Thus, the investment coat wrapping the ovigerous hair consists morphologically of the same materials as are present in the funiculus and the outermost layer of the egg envelope. The investment coat is tightly connected to the ovigerous
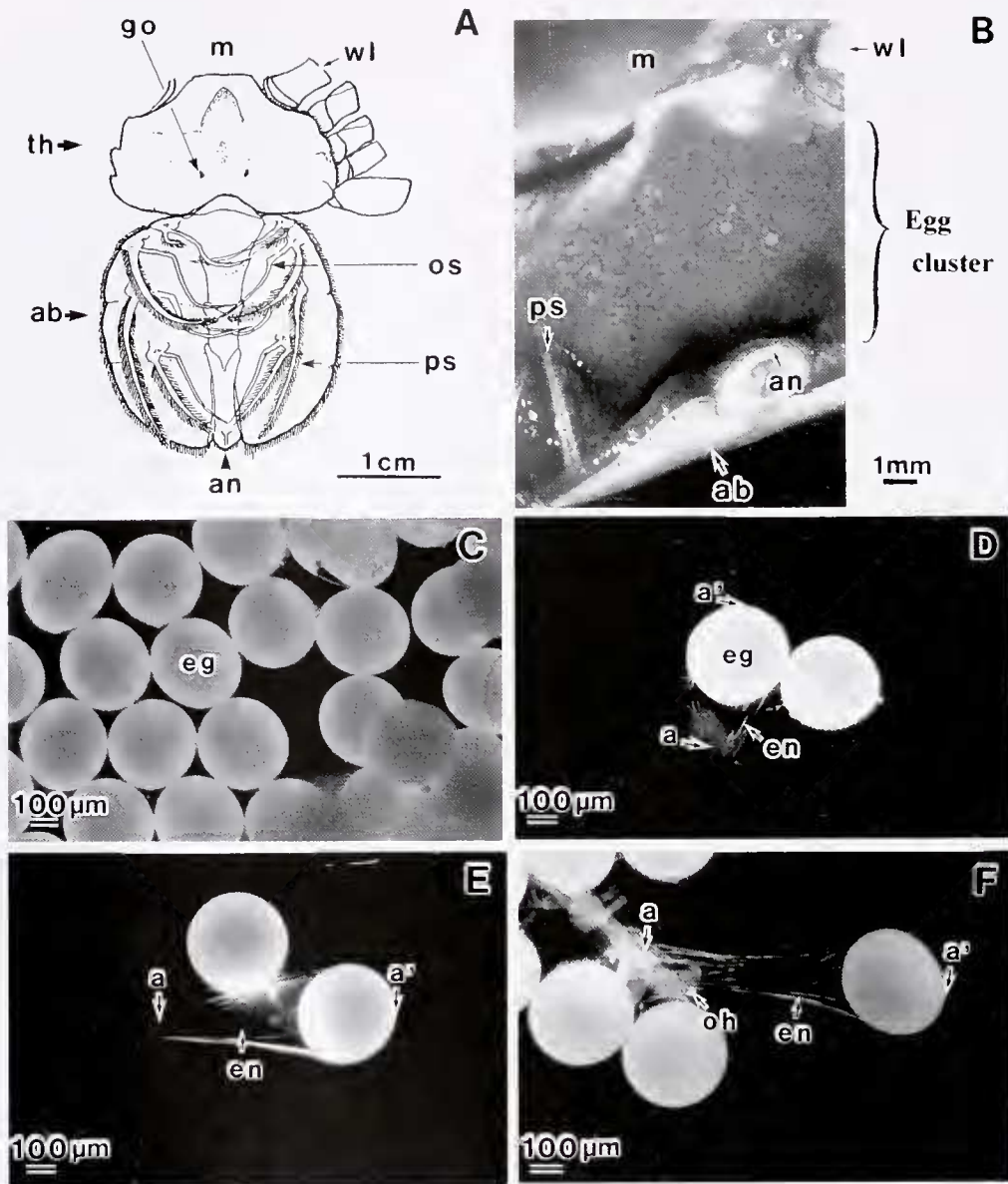
Figure 1. Egg-laying in *Sesarma haematocheir* and a method of measuring the adhesiveness and plasticity of the egg envelope. (A) Morphology of the thorax (th) and abdomen (ab) of a female. Eggs just extruded from the gonopore (go) attach to many ovigerous hairs (see Fig. 6A) arranged on the ovigerous seta (os). No eggs adhere to the plumose setae (ps). m: mouth parts. wl: walking leg. an: anus. (B) The incubation chamber, and an egg cluster newly extruded from the gonopore. The extruded eggs lie between thorax and abdomen (*i.e.*, incubation chamber), protected by the folded abdomen, and are incubated for nearly a month. The whole egg cluster is immersed in a transparent incubation fluid. Abbreviations are the same as in (A). (C) Eggs (eg) within 5 min after egg-laying. The egg envelope is not yet adhesive. (D) The eggs 20–30 min after egg-laying. The egg envelope (en) is adhering to the bottom of a petri dish. At this time, if an egg is gently rolled with a fine forceps, it moves a little, then it is slipped (or crushed) out of the envelope. *a*: one end of the envelope, where it adheres to the bottom of the petri dish (attachment point). *a′*: the other end of the envelope where the egg was rolled or pushed with a forceps. The plasticity of the envelope was measured as the length of *a–a′* at this time. (E) The egg 40 min after egg-laying. The envelope (en) showed both adhesiveness and plasticity. (F) The eggs 90 min after egg-laying. Adhesiveness of the envelope (en) was lost, so the envelope did not adhere to the petri dish. But the envelope attained maximum plasticity.

hairs (Fig. 3C); when clusters of embryos were pulled away from the ovigerous seta with fine forceps, most of the hairs broke away from the seta (not shown; see Saigusa, 1995).

Investment coat. When cross and oblique sections of the attachment were examined (Fig. 4A), it was always sublayer E1a that was seen to adhere directly to the
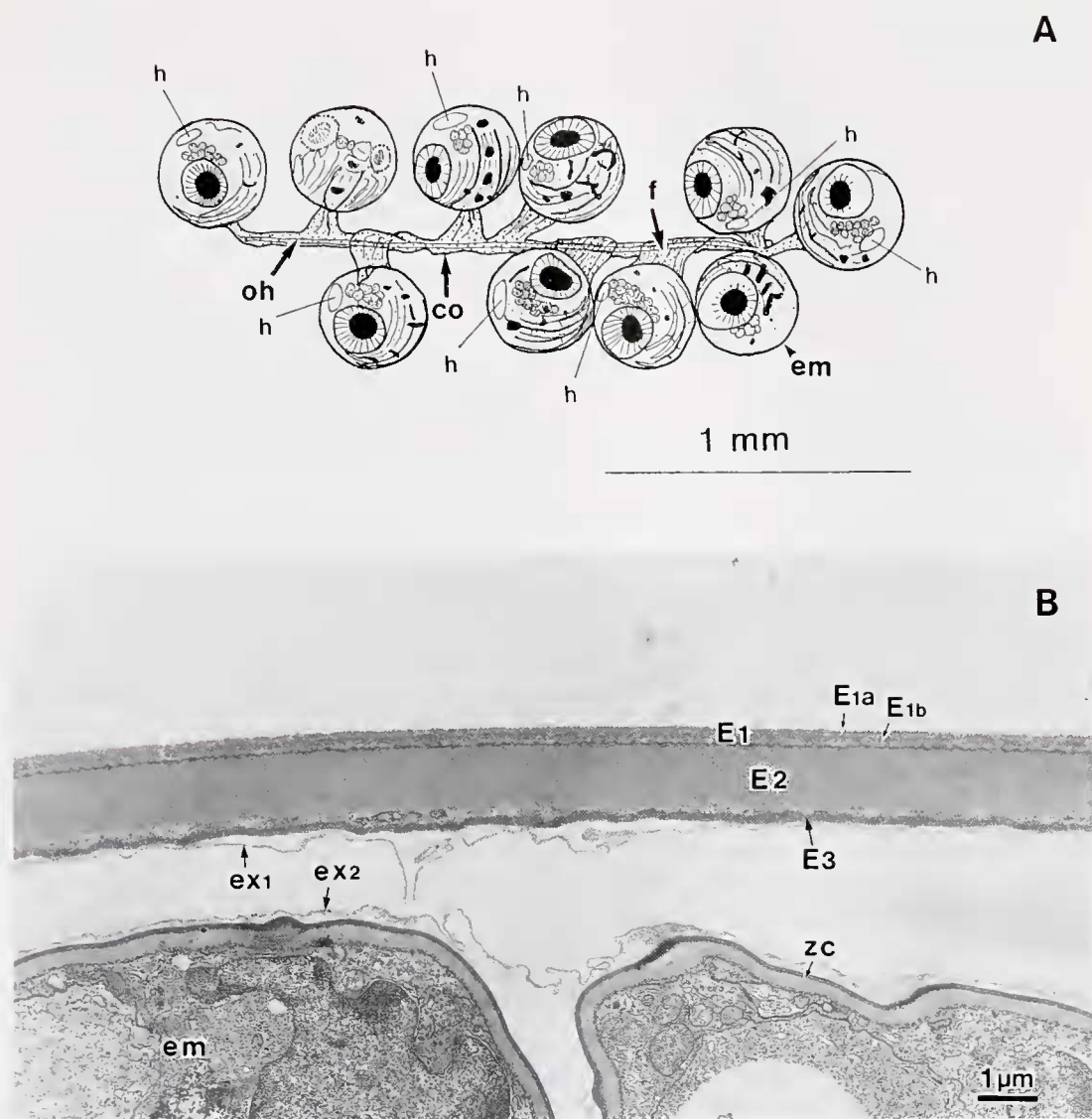
Figure 2. Embryo attachment system of *Sesarma haematocheir:* mature embryos that are within hours of hatching. (A) Connection of the embryo (em) to an ovigerous hair (oh). co: investment coat wrapped around the hair. f: funiculus. h: heart of each embryo. Note that the funiculus does not attach at a particular position with respect to the embryo. (B) Fine structure of the embryonic envelope. E1: the outermost layer, which consists of two further sublayers (E1a and E1b). E2: the middle layer. E3: the innermost layer. The embryo (em), close to hatching, is wrapped further in two very thin layers: *i.e.,* exuviae (ex1 and ex2) which are deposited by the embryo. zc: zoeal cuticle. Just after hatching, the zoeas cast off these exuviae and then begin to swim.

ovigerous hair (Fig. 4B, C). A cross section (Fig. 4B) showed the presence of a thin layer 40 nm thick (α1) on the surface of the ovigerous hair. This layer contacts ridges in E1a that are arranged parallel to the hair at intervals of 130–160 nm. Tubular structures, 50–70 nm in diameter, appear at random in the ovigerous hair (small arrows in Figs. 4B, 5A, B).

In oblique, nearly longitudinal section (Figs. 4C, 5A), the ridges on layer E1a in contact with the α1 layer appear as slender electron-dense structures arranged at the same interval as the ridges (130–160 nm) (compare Fig. 5A with

Fig. 4B: much the same magnification). In the oblique section, furthermore, the α1 layer is seen to be underlain by a comparatively electron-dense layer (α2). Many tubular structures (25–70 nm in diameter) were observed in this layer and just below it. The remainder of the cuticular wall of the hair has the usual stratified structures. Ovigerous hairs taken from female crabs before egg-laying were also examined (Fig. 5B). Note that iterated electron-dense structures are present on the surface of these hairs, and at the same intervals, although the investment coat, and thus E1a, are absent.
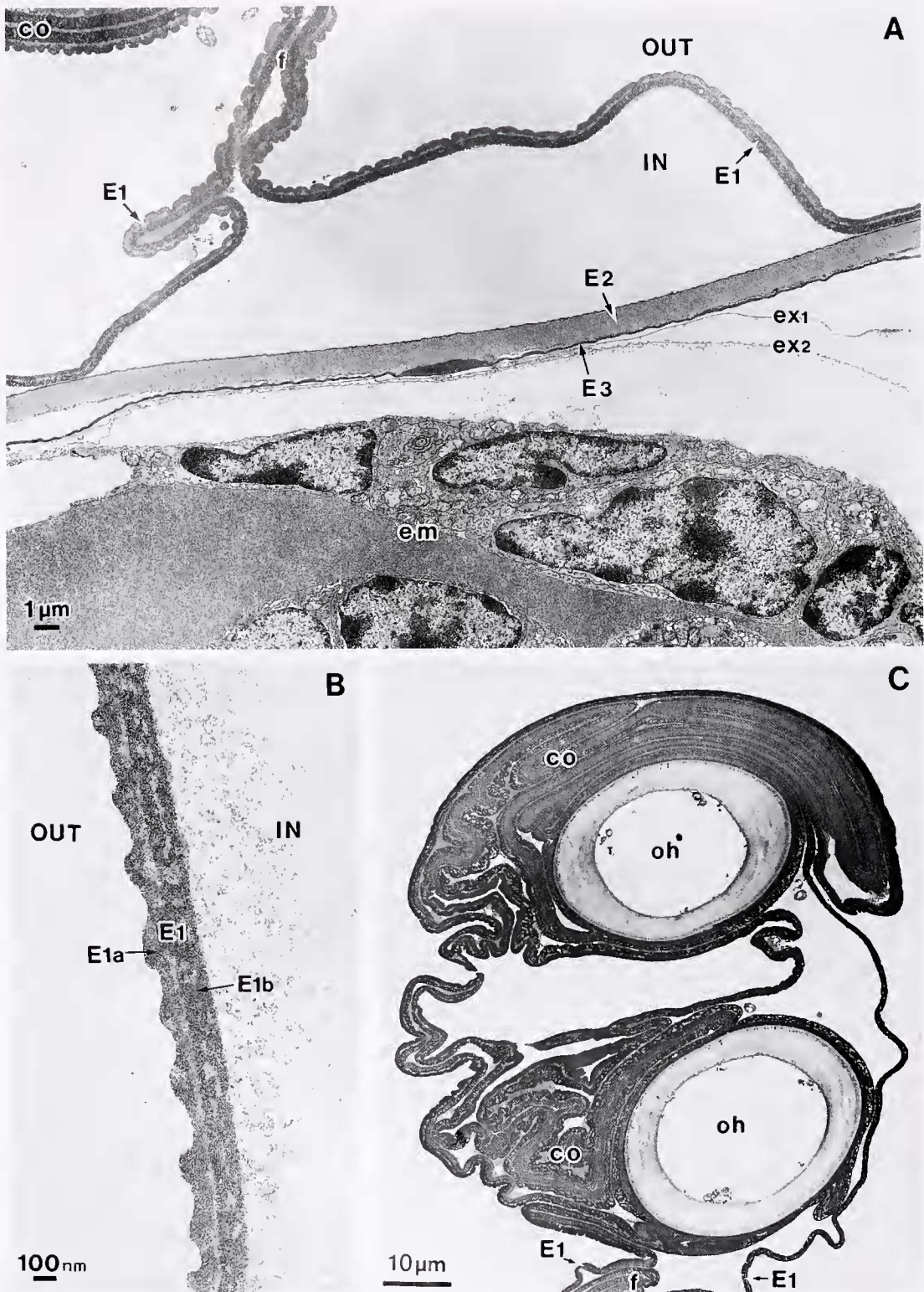
Figure 3. The components of the embryo attachment system: mature embryos that will hatch in a few days. (A) The funiculus (f) is composed of two opposed portions of egg envelope (E1). The funiculus extends toward the coat (co) wrapping one of the female's ovigerous hairs; the ovigerous hair is not shown. IN: inside the funiculus. OUT: outside the funiculus. E2 and E3: middle and innermost layers. em: embryo. ex1 and ex2: exuviae deposited by the embryo. (B) Components of the funiculus. E1a and E1b: two distinguishable sublayers of the outer layer E1. A substance of low electron density is seen inside the funiculus. (C) A continuous coat (co) investing two ovigerous hairs (oh). Symbols are the same as in (A).
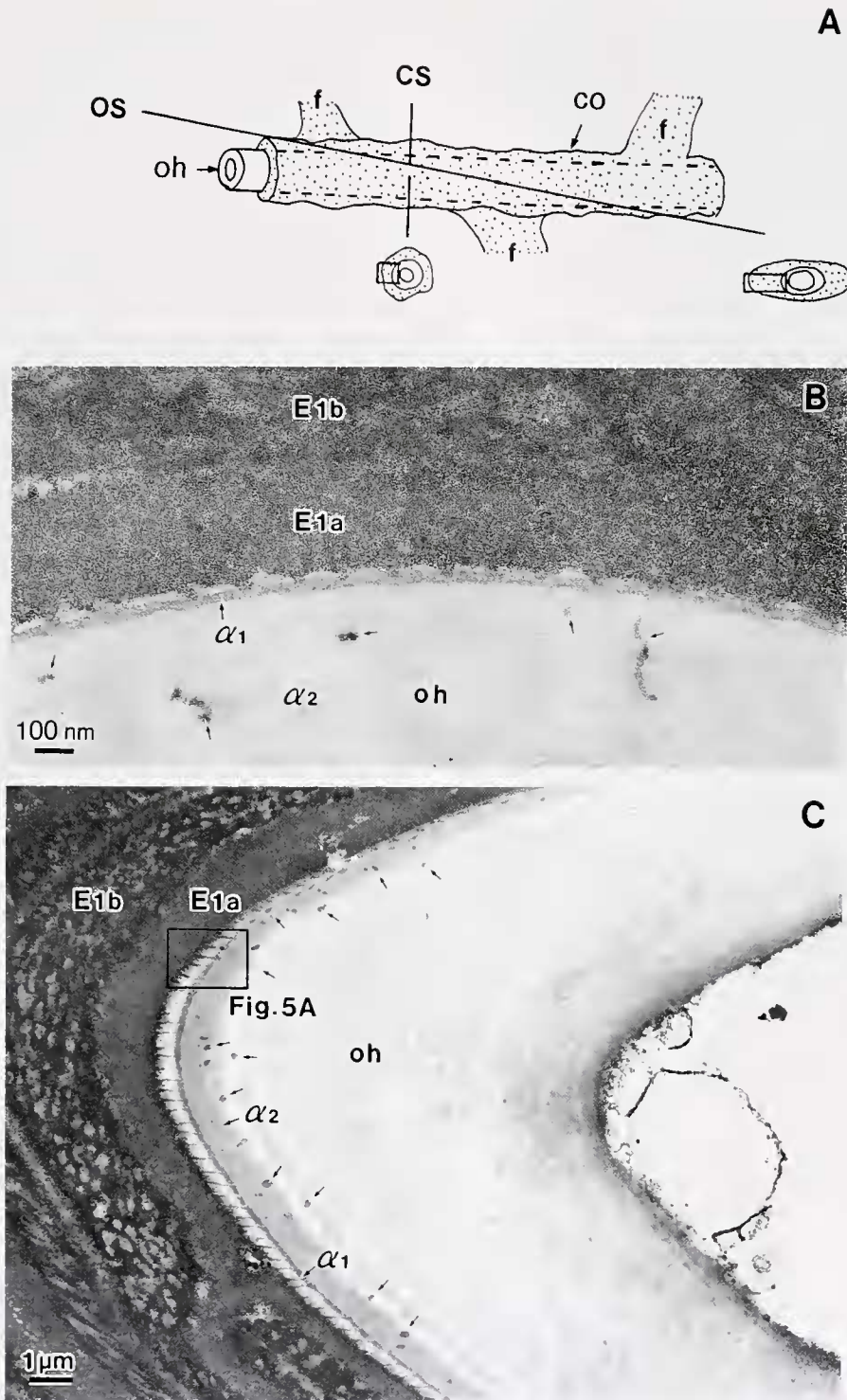
Figure 4. Attachment of the investment coat to the ovigerous hair; observed in mature embryos. (A) Sections of an ovigerous hair (oh) invested by the coat (co). CS: cross section. OS: oblique section, nearly longitudinal. f: funiculus. (B) In a cross section, ridge-like extensions of E1a are attached to a layer (α1) on the surface of the ovigerous hair. α2: a layered structure underlying α1. E1a and E1b are the two sublayers of E1. Small arrows indicate tubules in the α2 layer. oh: ovigerous hair. (C) In an oblique section (lower magnification), the ridges appear as slender, evenly spaced structures attached to sublayer E1a. Many tubular structures are seen in the α2 layer and just inside it (some of them are shown by small arrows). Compare with (B).
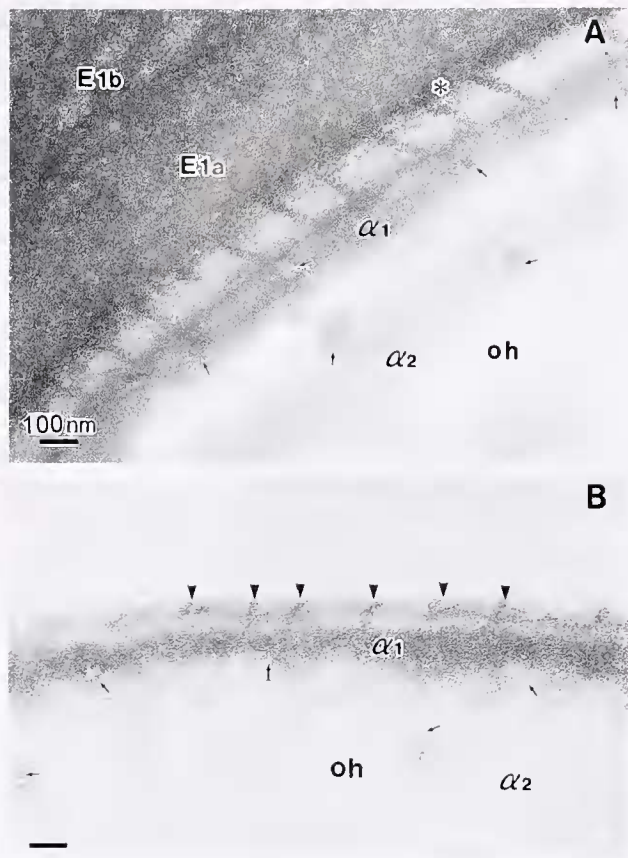
Figure 5. Structure of the attachment between the investment coat and an ovigerous hair. (A) An ovigerous hair (oh) wrapped in an investment coat (oblique section, magnified from Fig. 4C; the scale is the same as in Fig. 4B); observed in mature embryos. $\alpha 1$: the thin, electron-dense layer to which the slender extensions from layer E1a are regularly arranged. Small arrows show tubules. $\alpha 2$: a layered structure underlying $\alpha 1$. The thin, electron-dense layer (*) is also observed on the surface of E1a. (B) An ovigerous hair (oh) from a female that is not incubating a clutch of embryos. Thus, this hair is not invested by a coat. Note the electron-dense structures (downward solid arrow heads: ▼) about the same interval as the extensions from E1a (compare with A). Small arrows: tubules seen in both $\alpha 1$ and $\alpha 2$.

Formation of the embryo attachment system after egg-laying

Schedule of events. An examination of behavior in several females revealed the time course of egg-laying, egg attachment, and funiculus formation in *Sesarma*. No visible event signals the start of egg-laying, and it proceeds rapidly, so determining a precise schedule of this process was difficult. In addition, an exact point of transition between successive events could sometimes not be determined. Thus, the timing of each component behavior summarized in Table 1 involves some speculation.

The eggs are extruded from the gonopore and into the incubation chamber (Fig. 1B). Between 10 and 15 min seems to be required for all the eggs to be extruded. When the eggs are laid, the female leaves the water and stands motionless, with her walking legs stretched out and her abdomen half folded. At the same time, however, the pleopods—the ovigerous setae (Fig. 6A)—steadily knead the clutch. The eggs were never adhesive just after laying. Within 5 min, however, the volume of incubation fluid (a transparent liquid appearing in the incubation chamber) increases, and the egg cluster is immersed in this fluid (Fig. 1B). At this time, the egg envelope becomes strongly adherent to the ovigerous hairs (Fig. 6B). The eggs never attach to the plumose hairs (Fig. 6A).

Between 10 and 20 min after egg-laying, the female is still motionless with her abdomen half open, and the egg cluster is still being kneaded by the ovigerous seta. But the number of eggs adhering to the ovigerous hairs is markedly increased. While the eggs are attaching to the hairs, the egg envelope is expanding; its volume becomes 1.5–1.6 times larger than that of the egg itself (Fig. 6C), and it is still strongly adhesive (Fig. 6D). Although the investment coat is complete between 10 and 40 min after egg-laying, the funiculus is not yet formed at this time.
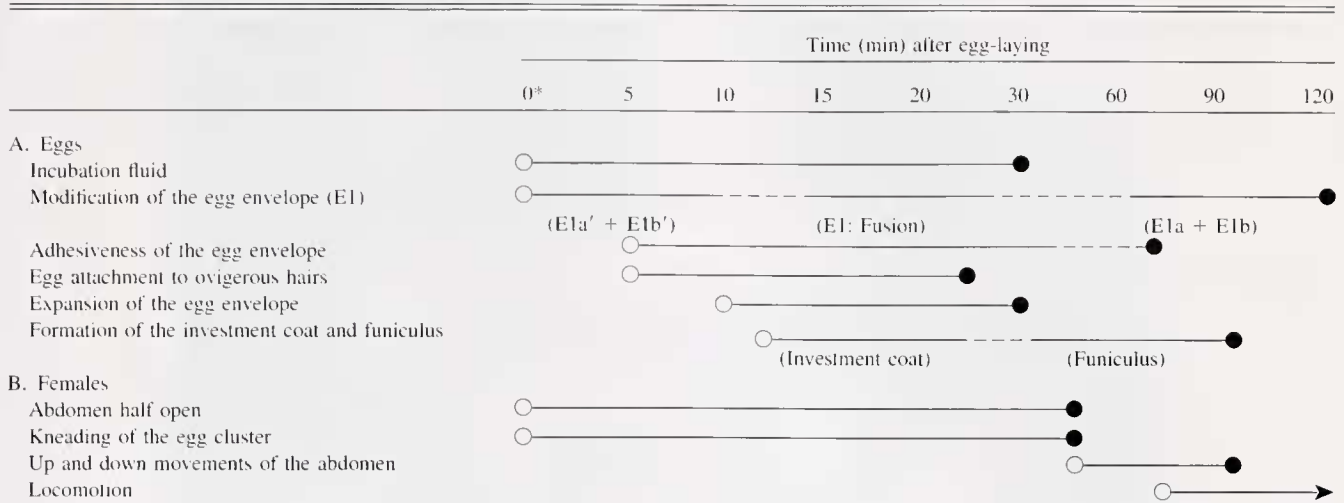
Forty minutes after egg-laying, adherence of the egg envelope seems, actually, to decrease; that is, the eggs still adhere to the bottom of the petri dish (see Fig. 9), but the attachment point (*a* in Fig. 1E) shifts slightly when the egg is gently pushed with a forceps. The incubation fluid immersing the egg cluster is also decreased 40 min after egg-laying. The funiculus begins to form at about this time (Fig. 6E), and is completed between 60 and 120 min after egg-laying. With the funiculus formed, the abdomen of the female begins to move up and down, but this movement stops 2 h after egg-laying. With this behavior, all the eggs would be well folded by the abdomen. This brief period of abdominal fanning is not likely to aerate the eggs; possibly, it serves to fold the clutch into the incubation chamber. At this time, the female becomes mobile again, and can easily escape in response to external stimuli.

Figure 6F shows eggs that were kept in 20 mM Tris-HCl buffer in a petri dish for one night after egg-laying. At first, the eggs adhered to the bottom of the dish, or to the other eggs. In the morning, the diameter of the envelope had expanded 3-fold. These eggs could be detached with forceps from the dish or from the other eggs without envelope deformation, but such eggs were never again attached to either a dish or to the other eggs. The plasticity of the envelope was also lost.

Egg envelope: origin of the outermost layer (E1) and its morphological change after egg-laying. The fine structure of a mature, unfertilized egg is shown in Figure 7A. The egg is enclosed by a follicle cell (about 1 μm thick), beneath which is an envelope (vitelline membrane) consisting of two sublayers (E1a' and E1b'). The sublayer E1b' is about 0.5 μm thick in the ovary (Fig. 7A); but within 5–10 min after egg-laying, E1b' becomes 5-fold thicker (2.5 μm; Fig. 7B).

Table 1

Time course of egg attachment and formation of the investment coat and funiculus

	Time (min) after egg-laying								
	0*	5	10	15	20	30	60	90	120
A. Eggs									
Incubation fluid	○———————————————●								
Modification of the egg envelope (E1)	○— — — — — — — — — — — — — — — — — — —●								
		(E1a′ + E1b′)		(E1: Fusion)				(E1a + E1b)	
Adhesiveness of the egg envelope		○— — — — — — — — — — — — — —●							
Egg attachment to ovigerous hairs		○————————————●							
Expansion of the egg envelope			○—————————●						
Formation of the investment coat and funiculus			○— — — — — — — — — — —●						
			(Investment coat)				(Funiculus)		
B. Females									
Abdomen half open	○————————————————————————●								
Kneading of the egg cluster	○————————————————————————●								
Up and down movements of the abdomen							○————————●		
Locomotion							○——————————————➤		

* Zero minutes indicates the estimated end of egg-laying.
Symbols: ○— start, —● end, —➤ continuing, — — — — uncertain limits of sequential events.

The sublayer E1b′ is further modified, as its structure comes to resemble that of sublayer E1a′ (Fig. 7C). Soon the distinction between the two sublayers is completely lost, and they fuse to form a homologous envelope (1.2 μm thick); we designate this layer E1 (Fig. 7D). The intermediate stage in the formation of E1 (Fig. 7C) was rarely observed, indicating that the process of fusion progressed very rapidly, possibly within 5 min after the thickening of E1b′. The cause of the rapid modification of sublayer E1b′ is not known, but it could be related to the appearance of clusters of electron-dense granules that seem to be secreted from the egg into the spaces below E1b′ (arrows in Fig. 7B).

After the fast modification of E1b′, the homogenous structure of layer E1 is retained for 10–30 min after egg-laying. By 30–60 min after egg-laying, E1 again divides into two sublayers, E1a and E1b (Fig. 7E); but whether these new sublayers correspond to the original E1a′ and E1b′ (Fig. 7B) is not known. In any event, our morphological findings suggest that the outermost layer enveloping the egg (E1) is produced by the egg itself, rather than being formed by externally secreted materials. Also, formation of the middle layer (E2) begins at 10–15 min after egg-laying (Fig. 7D).

Formation of subsequent layers of the egg envelope. In addition to the outermost layer (E1), a thick middle layer (E2) and an innermost thin layer (E3) invest the embryo (Fig. 2B). These two layers are included in neither the funiculus nor the investment coat. The middle layer (E2) appeared at about the time that E1 was becoming homogeneous in structure—possibly 10–15 min after egg-laying (Fig. 7D). The middle layer forms very slowly, and is complete 12–24 h after egg-laying (not shown). This layer may be formed by the secretion from the embryo (see arrows in Fig. 7D, E). The third (E3) layer appears a few days later, possibly secreted by the egg like the middle layer (not shown).

The egg envelope attaches to ovigerous hairs. As mentioned earlier, the egg envelope expands just after attaching to the ovigerous hairs (Figs. 6C, 8A). The interaction between the envelope and the ovigerous hair was examined by transmission electron microscope. Attachment of the envelope onto the ovigerous hair occurred about 10 min after egg-laying, when two sublayers (E1a′ and E1b′) began to fuse (Fig. 8B). At higher magnification (Fig. 8D), the egg envelope can clearly be seen to be a homologous layer (E1) (see also Fig. 7D).

The egg envelope at this time (10–15 min after egg-laying: Fig. 8A–D) is strongly adhesive, so it adheres, not only to the hair, but also to other envelopes. For example, Figure 8B shows an envelope (E1) folded on the upper half of the hair. While this envelope is adhering to the ovigerous hair for 14–15 μm (from points a1 to a2), it is also adhering to itself for 20 μm (b1–b2). Note that the envelope in Figure 8B is not attached to the lower part of the hair; this may be due to a weak attachment that was separated during fixation or embedding. The large space between E1 and E2 suggests that the envelope (E1) had started to expand or elongate.

The connection between the newly attached homogenous layer (E1) and the ovigerous hair (Fig. 8C and 8D) is similar to that in a mature coat (Fig. 5A). In Figure 5A, however, the surface of E1a appears as an electron-dense
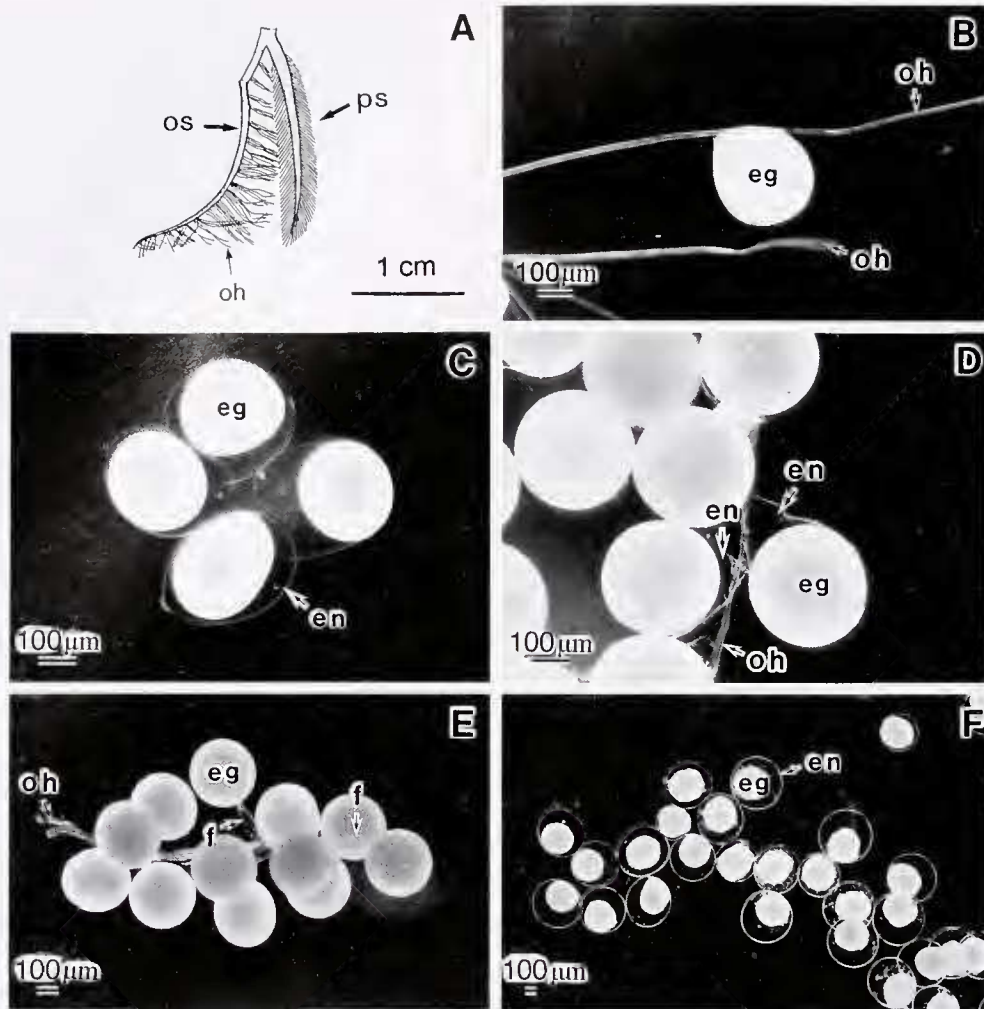
Figure 6. Egg attachment and formation of the funiculus after egg-laying; observed with a stereomicroscope. Observations of the eggs that were placed in a petri dish just after laying. (A) A pair of abdominal appendages of a female: ovigerous seta (os) and plumose seta (ps). Ovigerous hairs (oh) are arranged on the ovigerous seta. (B) An egg (eg) adhering to an ovigerous hair (oh): 5–10 min after egg-laying. (C) Expansion of the envelope (en): 10–15 min after egg-laying; the envelope shows strong adhesiveness. (D) Formation of the *investment coat* following attachment to the hair: 15–30 min after egg-laying. (E) Formation of *funiculus* (f): 60 min after egg-laying. Although the adhesiveness of the envelope is lost, the plasticity increases. (F) Eggs placed in a petri dish containing 20 m*M* Tris-HCl buffer overnight. Egg envelope swells in all embryos. Adhesiveness of the envelope has already been lost.

thin layer distinguishable from another sublayer (E1; 40–50 μm thick in oblique section). Such a layer-like structure was never observed in the newly attached envelope. Figure 8E shows the egg envelope about 80 min after egg-laying. The funiculus has already been formed. Moreover, the envelope and funiculus are again divided into two sublayers (E1a and E1b), and the inner layer (E2) has thickened.

Temporal changes in egg envelope adhesiveness and plasticity. Newly extruded eggs were removed from the female every 10 min after egg-laying. The eggs were placed in a polystyrene petri dish, and temporal changes in the adherence and plasticity of the envelope were examined

with time. Adherence was estimated with 10–20 eggs, and plasticity was measured with 5–10 eggs (Fig. 9). The egg envelope was not adhesive immediately after egg-laying (not shown), but after 5 min, the envelope became strongly attached to the bottom of the dish, and it did elongate as soon as it was gently rolled with a fine forceps.

In Figure 9, adherence of the egg envelope was 100% between 10–50 min after egg-laying. But 40–60 min after egg-laying, the fixed attachment point (*a* in Fig. 1E) became mobile when the eggs were rolled with a forceps. Thus, envelope adhesiveness begins to decrease markedly about 40 min after egg-laying. In contrast, envelope plasticity
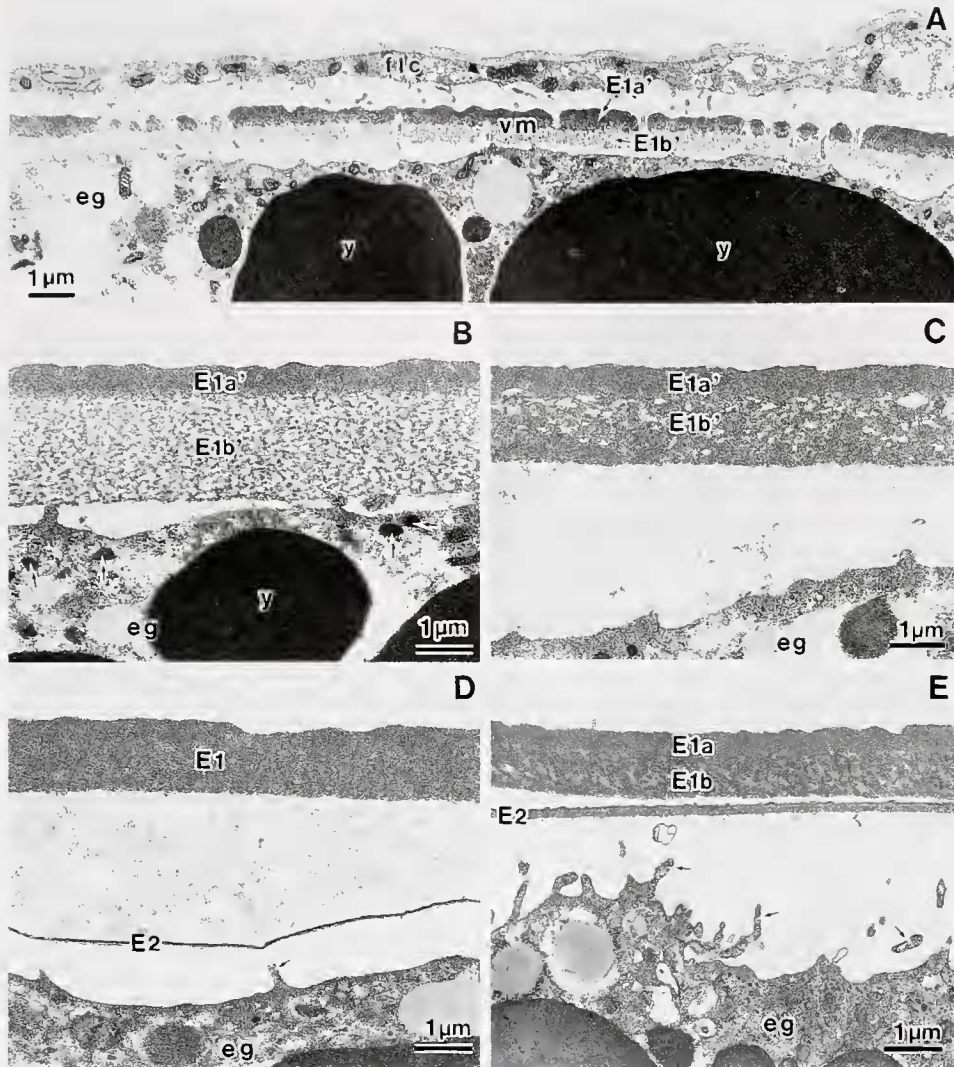
Figure 7.   Origin of the egg envelope and morphological changes that occur after egg-laying. (A) Surface layers investing a fertilized, mature egg (eg) in the ovary. E1a′ and E1b′: two sublayers of the envelope, *i.e.*, vitelline membrane (vm). flc: follicle cell. y: yolk. (B) The egg envelope 5–10 min after egg-laying. Electron-dense materials seem to be secreted by the embryos (small arrows). (C) The egg envelope about 10 min after egg-laying. Sublayer E1b′ begins to fuse to E1a′. The envelope is adhesive, but plasticity is very weak. (D) The egg envelope 10–30 min after egg-laying. The sublayers of E1 are not distinguishable, and plasticity increases. Small arrows show a possible secretion from the egg. The middle layer (E2) begins to form. (E) The envelope 80 min after egg-laying; sublayers have reappeared. The expanded envelope becomes the funiculus. E2 becomes thicker. Small arrows: possible secretion from the embryo to make E2.

reached a first peak at 40 min after egg-laying and then decreased until 70 min after egg-laying. But at this time, plasticity again increased rapidly, attaining another peak 90 min after egg-laying. After 90 min a stretched envelope (Fig. 1F) regained its form somewhat when the tension was released. Over the next few hours, envelope plasticity gradually decreased again. The size of the eggs did not change through the attachment process.

Egg attachment to transplanted setae. Two segments of an ovigerous seta were removed from a non-incubating female and inserted among the egg clusters of another

female that had been laying eggs. The donated segments were removed from the host female after 5 min and after 1 h, and examined with a stereomicroscope. The setal segment removed after 5 min had nearly 50 eggs attached to its hairs (Fig. 10A), and the attachment was similar to those of intact females (Figs. 6B and 8A). However, neither the coat nor the funiculus were formed, and the egg envelope was not expanded at this time. The segment removed after 1 h had many eggs attached to its hairs (Fig. 10B). Since adherence of the envelope has already been lost by 1 h (Table 1), the short structure attaching each egg to the hair must be
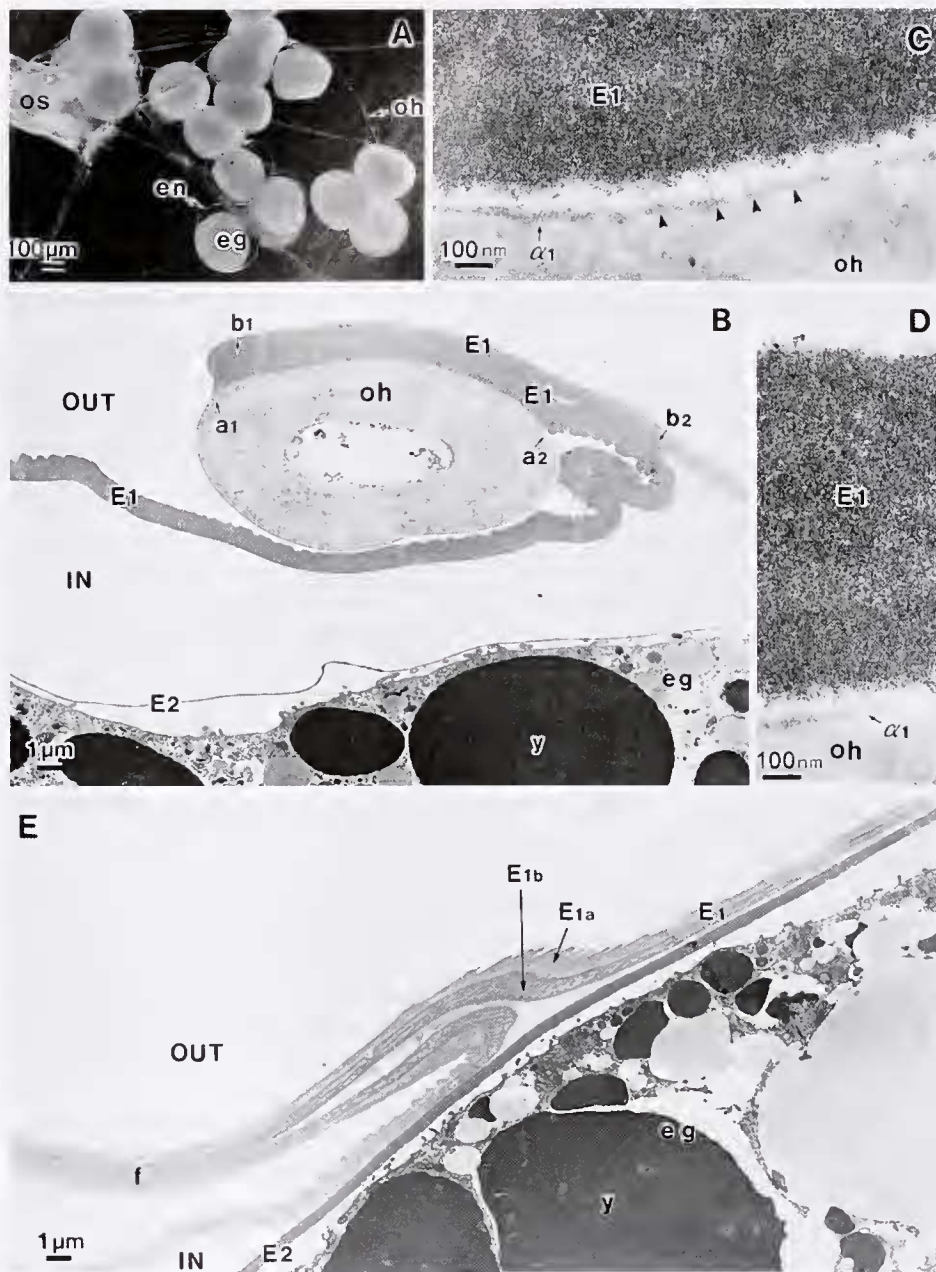
Figure 8. Attachment of eggs, and formation of the investment coat and the funiculus. (A) Eggs (eg) 10–15 min after egg-laying. en: envelope. os: ovigerous seta. oh: ovigerous hair. (B) Fine structure of the envelope attaching to the hair: 10–15 min after egg-laying. eg: egg. y: yolk. E1: a homogeneous layer formed by the fusion of E1a and E1b. This layer wraps around the hair or is stratified on the hair (oh), and thus produces the coat. IN: inside E1. OUT: outside E1. a1–a2: envelope-hair adherence. b1–b2: envelope–envelope adherence. E2: the middle layer; its formation has just started. (C) Attachment of fused layer (E1) to the walls of an ovigerous hair (oh): 10–15 min after egg-laying (oblique section). Upward arrowheads show the electron-dense structures about the same interval on the hair. $\alpha1$: an electron-dense surface layer of the hair. (D) The whole envelope (E1) is homogeneous at this time: oblique section. (But very careful inspection indicates that E1a is still distinguishable from E1b even when the fusion occurs. See also Figures 7D and 8C.) (E) Completion of the funiculus (f): The egg about 80 min after egg-laying. E1 is again divided into two distinguishable sublayers E1a and E1b. E2 becomes thicker, but E2 is not a component of the funiculus. Other symbols are the same as in (B).
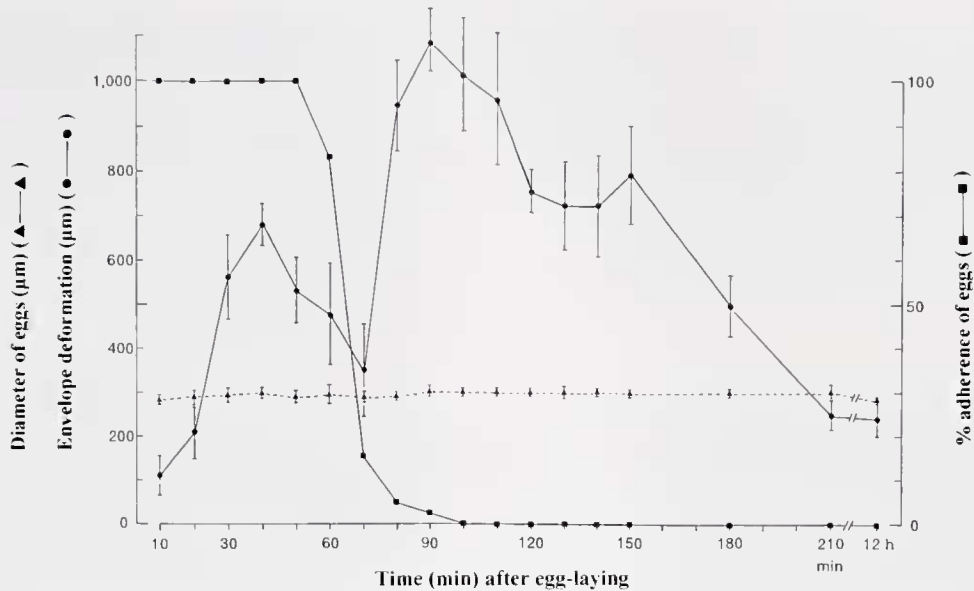
Figure 9. Time course of changes in the adhesion and plasticity of the envelope after egg-laying. Envelope adhesion (solid squares: ■), plasticity (solid circles: ●), and diameter of the eggs (solid triangles: ▲) were determined every 10 min. Data points are means with standard deviation (vertical bars).

an immature funiculus (Fig. 10C1–C3). These three examples also show that the hair was incompletely invested by the coat. In summary, the morphology and length of the funiculus were different from those formed in intact females. These funiculi (Fig. 10C1–C3) on the donated setae were shorter and wider than those of intact females (*e.g.*,
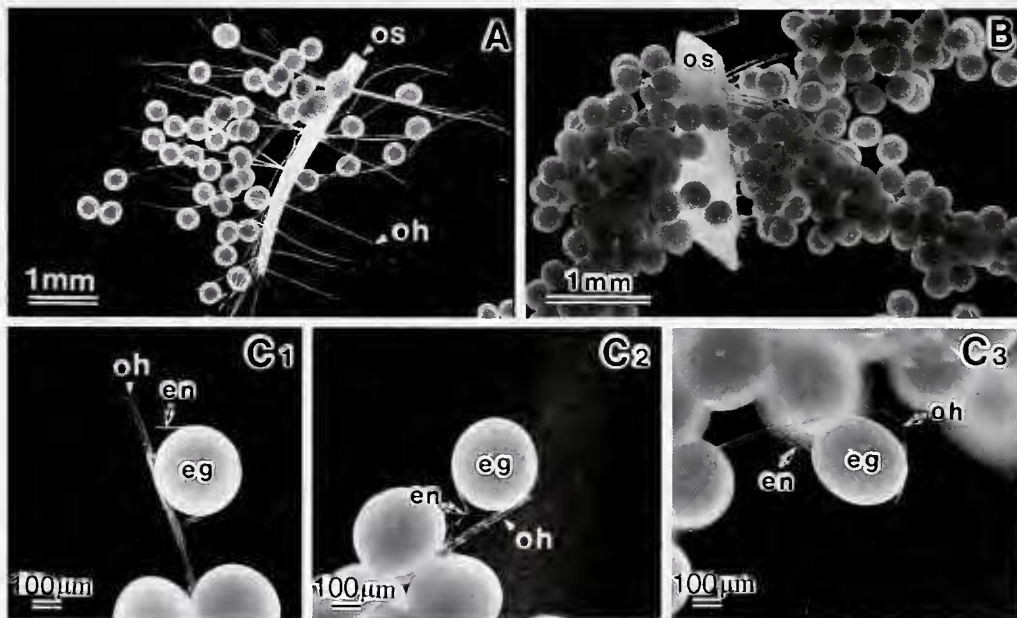

Figure 10. Segments of ovigerous setae were removed from non-incubating females and inserted into the egg mass of a female immediately after egg-laying. Effects on egg attachment and funiculus formation were observed. (A) A transplanted segment of an ovigerous seta (os) removed from host female 5 min after egg-laying. oh: ovigerous hair. (B) A transplanted segment removed 60 min after egg-laying. (C) Three examples of the incomplete attachment system formed on the ovigerous hair (oh) in (B). eg: egg. en: an envelope that has already lost adhesiveness.
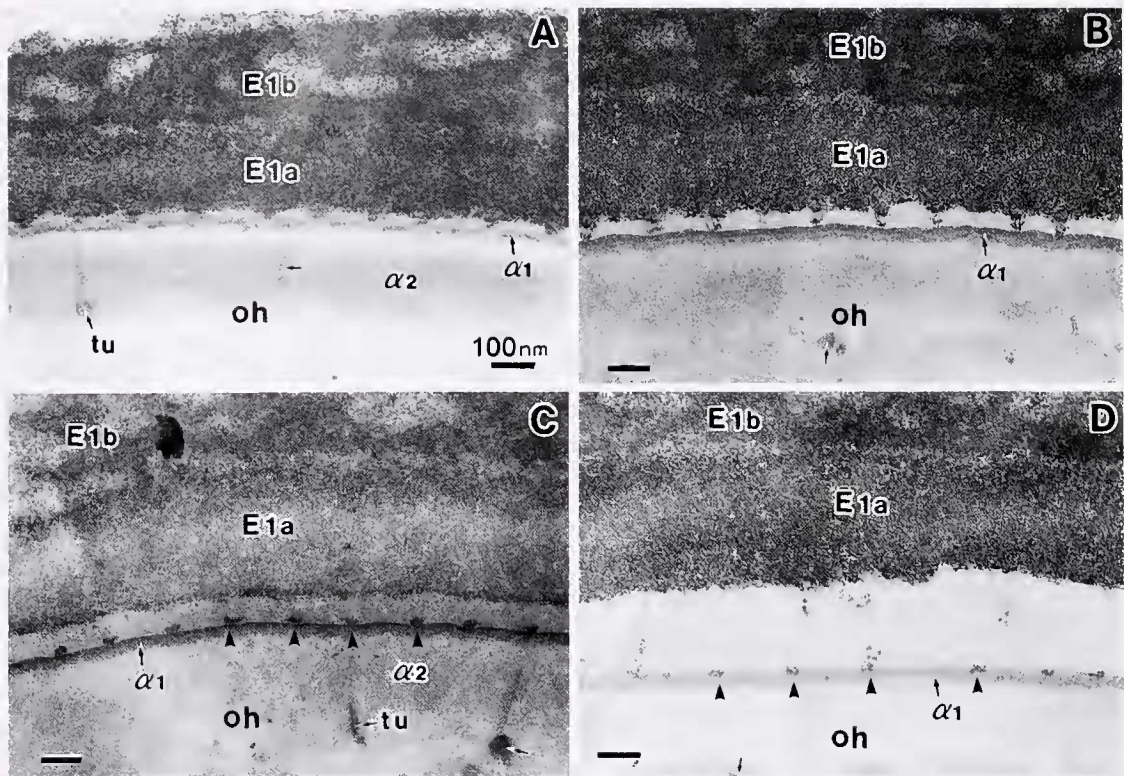
Figure 11. Time course of the dissociation of the investment coat from the ovigerous hair. Clusters of embryos were incubated with crude, concentrated hatch water for 2 min (A), 4 min (B), 10 min (C), and 120 min (D). E1a and E1b: sublayers of the investment coat. oh: ovigerous hair. α1 and α2: surface layers of the hair. Arrows: tubules (tu). Note that the extensions of the sublayer E1a retract, but electron-dense sites remain in the surface of the α1 layer of the ovigerous hair (upward arrowheads in C and D). Scales are all 100 nm.

Figs. 2A and 6E). We speculate that the kneading of the egg cluster by the ovigerous setae was not fully transferred to the donated setal segment, so the investment coat and funiculus were incomplete.

Disposal of the embryo attachment system after hatching

The outermost layer of the egg envelope (E1a) attaches to the ovigerous hair (Fig. 4). Moreover, electron-dense, slender structures arranged at intervals of 130–160 nm around the hair seem to be involved in attachment. Figure 11A shows a section of a hair in an investment coat; the hair was treated with crude, concentrated hatch water and fixed 2 min later. The sublayer E1a is still adhering to the ovigerous hair through its ridges. In a section of a hair fixed 4 min after the treatment with hatch water (Fig. 11B), the space between the hair and the envelope has become slightly wider, but the connection between the hair and the ridges of E1a still remain. Note, however, that some of the ridges on E1a seem to be dissociating from a structure that remains on the hair. After 10 min of treatment with hatch water, the envelope was completely detached from the hair, and the dissociation of the ridges of E1a from the structures on the hair has

advanced (Fig. 11C). Two hours after treatment (Fig. 11D), the space was widely expanded, and the ovigerous hair easily slipped out of the coat, when the embryos were pulled with a fine forceps. The surface of the stripped hair in Figure 11D is strikingly similar to the hair of a non-incubating female (Fig. 5B). No clear changes were observed in other parts of the investment coat (*e.g.*, compare Fig. 4B with Fig. 11D) or the funiculus (not shown).

Discussion

The embryo attachment system of the estuarine terrestrial crab *Sesarma haematocheir* consists of (1) the egg envelope, (2) the funiculus, and (3) the investment coat, which wraps around an ovigerous hair. Our observations show that the entire embryo attachment system originates from the vitelline membrane that surrounds an unfertilized egg (ovum) in the ovary. Furthermore, as the adhesiveness and then the plasticity of this envelope change, the sublayers E1a′ and E1b′ fuse and then reappear, and the investment coat and then funiculus are formed. These findings are first compared with the structure and formation of the embryo attachment system in other decapod crustaceans. Then, we

propose that orderly production of the egg attachment system—particularly the investment coat and the funiculus—is dependent upon a series of correlated changes in the mechanical properties of the egg envelope. Furthermore, we suggest that, after the larvae have hatched, OHSS acts specifically at the attachment sites, disposing of the embryonic attachment system and preparing the ovigerous hairs for a new clutch of eggs: that is, the ovigerous hair recycling system in these crabs is proposed.

Structure and formation of the egg attachment system: comparison with other crustaceans

Under transmission electron microscopy (TEM), the mature embryonic envelope of *S. haematocheir* consists of three layers (E1, E2, and E3) (Fig. 2). Cheung (1966) observed the mature embryonic envelope of the crab *Carcinus maenas* with a the light microscope (LM), and reported that it consists of three layers which are histochemically distinct (layers 1–3). But it seems that the layers reported by Cheung (1966) do not exactly correspond to the three layers (E1–E3) we have reported here. Envelope formation in *C. maenas*, observed under TEM, was reported by Goudeau and Lachaise (1980). The ultrastructure of the outermost layer (E1) and the thick middle layer (E2) is much the same as that of *S. haematocheir* (Fig. 2). Furthermore, a fertilized egg that has just been extruded into the incubation chamber of the female contains a single envelope (E1) with two sublayers, and the entire embryo attachment system originates from the vitelline membrane that surrounds an unfertilized egg (ovum) in the ovary. This result is also the same as in *C. maenas* reported by Goudeau *et al.* (1987). These findings are contrary to previous notions that a portion or all of the embryo attachment system is formed by extra-ovarian secretions from "cement glands" in the maternal pleopods (see Yonge, 1937, 1955; Stephens, 1952; Aiken and Waddy, 1982; Fisher and Clark, 1983; Saigusa, 1994, 1995).

In the lobsters *Homarus americanus* and *H. gammarus*, the envelope of the newly extruded eggs is also single, and this envelope swells and is adhesive after egg-laying; the envelope is stretched by a vigorous beating of the female pleopods, producing the funiculus (Goudeau *et al.*, 1987). A similar mechanism was also reported for the embryo attachment system of *C. maenas* (Goudeau and Lachaise, 1980). Formation of the embryo attachment system of *S. haematocheir* is also similar to that of other decapods (Cheung, 1966; Goudeau *et al.*, 1987; Talbot and Goudeau, 1988). Thus, the embryo attachment system of most decapod crustaceans may be formed by the same fundamental mechanism.

Newly extruded eggs of *S. haematocheir* are immersed in a transparent incubation fluid until the funiculus starts to form (Fig. 1B). A forceps was put in the incubation chamber, and gently pulled at the surface of the fluid. The viscosity was measured by the distance at which the pulled thread of fluid detached from the bulk fluid in the incubation chamber. Although estimation of this distance could only be rough, this method indicated that the viscosity of this incubation fluid is not different from that of water (unpubl. obs.). Cheung (1966) reported that cement glands are present in *Astacus*, and that a mucous substance may be secreted into the abdomen at egg-laying; but he also noted that such glands are not found in *Carcinus*. We speculate that the incubation fluid may not be a "cement substance," but only water generated by the action of the maxillipeds at the mouth.

Formation of the embryo attachment system associated with changes in the mechanical properties of the egg envelope

Just after fertilization, the vitelline envelope of crustaceans is transformed into the fertilization envelope by the cortical reaction (*e.g.*, Pillai and Clark, 1990; Cuoc *et al.*, 1994). In *Sesarma haematocheir*, the expansion of layer E1b′ may be produced by this reaction, just after egg-laying (Fig. 7B). The expansion of this layer is the first noticeable morphological change (Goudeau and Lachaise, 1980; Talbot and Goudeau, 1988). This envelope then becomes adhesive at the same time.

As shown in Table 1 and Figure 7C, fusion of layers E1a′ and E1b′ begins 5 min after egg-laying, and E1a and E1b appear again 20–30 min after egg-laying. Adhesiveness may be produced by the fusion of these layers, although the mechanism of fusion is not known yet. When the eggs at the bottom of a petri dish were gently pushed with the forceps between 5 and 20 min after egg-laying, the envelope—being strongly pasted on the bottom of the dish—was immediately broken, and the egg was either crushed or it escaped from the envelope. But 20–40 min after egg-laying, the envelope could be easily elongated because its plasticity increased. Thus, we speculate that the adhesiveness of the egg envelope is actually strongest 5–20 min after egg-laying, and that all the eggs attach to the ovigerous hair at this time. The female kneads the egg cluster after egg-laying (Table 1). This behavior would enhance the number of contacts between the egg envelope and the ovigerous hair, and thus help attachment to the hair.

On the other hand, the envelope plasticity increases 20–30 min after egg-laying (Fig. 9). This means that the egg envelopes attached to the ovigerous hairs are elongated by the movement of the maternal ovigerous seta. While the adhesiveness of the envelope still remains strong (20–30 min after egg-laying), an envelope would attach to envelopes at other sites. Movements of the ovigerous seta would
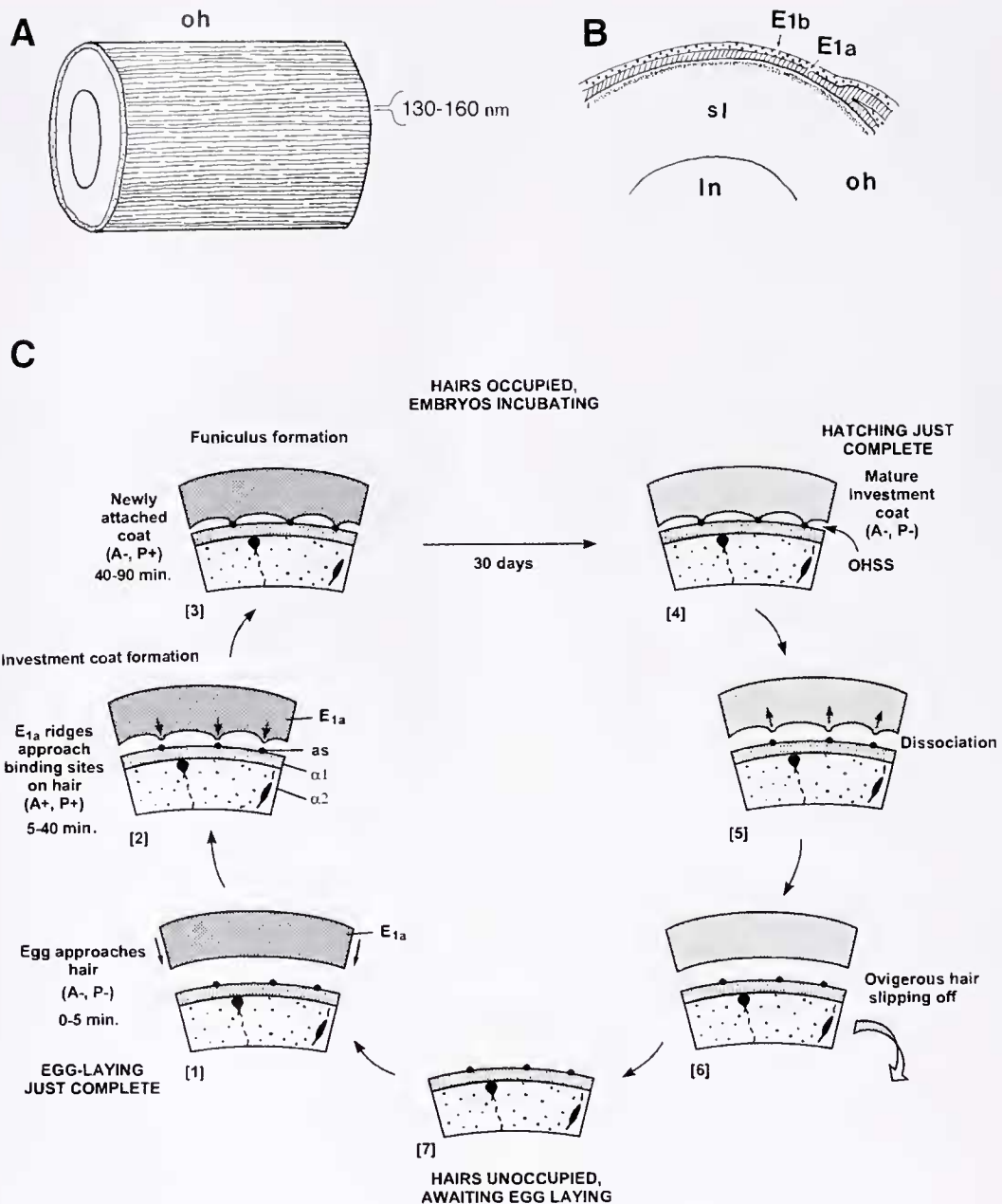
Figure 12. Attachment structures on the surface of the ovigerous hair and the egg envelope. (A) Diagram of an ovigerous hair (oh) that is not occupied by an investment coat. Proposed linear attachment structures are arranged around the circumference of the hair at intervals of 130–160 nm. (B) An investment coat wrapping around an ovigerous hair (oh). E1a and E1b are two sublayers of the coat. sl: stratified layering of the cuticle. ln: central lumen of the ovigerous hair. (C) Schematic of ovigerous hair recycling. [1] A few minutes after egg-laying: an egg envelope approaches a cleaned ovigerous hair (arrows). But the envelope is not yet adhesive. [2] Five min later the envelope shows adhesiveness. The outer layer of the adhesive envelope E1a develops ridges that extend (small arrows) toward attachment sites (as) on layer α1, which is on the surface of the hair. [3] The E1a ridges bind to the attachment sites and, through the action of the pleopods, an investment coat forms. After the funiculus forms (not shown), the embryos are incubated for nearly 1 month. [4] The embryos hatch, and OHSS (ovigerous-hair stripping substance) attacks the binding of E1a to the attachment sites on the hair. [5] As the investment coat dissociates from the hair, ridges on layer E1a retract, and [6] the investment coat slips off. The female picks remnants of the embryo attachment system off her hairs, and [7] the unoccupied hair is now ready for the next batch of eggs.

wind the elongated egg envelope around the hairs or layer the envelope on the hair. Since the envelope is still strongly adhesive until 40 min after egg-laying, the layers of envelope thus coiled or folded on the hair would adhere to each other, producing the investment coat. While the adhesiveness decreases 40 min after the egg-laying, envelope–envelope adhesion would not persist. In contrast, envelope plasticity remains (Fig. 9). So the envelope 40 min after the egg-laying would form the funiculus.

Embryos of some decapod crustaceans—for example, lobsters in the genus *Homarus* (Yonge, 1937; Talbot and Harper, 1984) and shrimps in the genera *Palaemonetes* (Burkenroad, 1947) and *Palaemon* (Fisher and Clark, 1983)—attach, through the funiculus, not only to ovigerous hairs but also to other embryos. In *S. haematocheir*, however, embryo-to-embryo attachment was only rarely observed. This could be easily explained by changes in adhesiveness and plasticity of the egg envelope after egg-laying. As shown in Figure 8A, when the egg envelope is strongly adhesive, it would attach not only to the ovigerous hairs but also to the other eggs. When the egg cluster is kneaded by the female pleopods, the envelope would become the investment coat. Thereafter, the envelope adhesiveness decreased, whereas the plasticity increased greatly (Fig. 9). At this time, envelopes elongated by the movement of the pleopods would never attach to each other and would thus produce a single funiculus, which would not adhere to the other eggs. In other decapod crustaceans, kneading of the egg cluster may stop while the adhesiveness of the envelope still remains; or the plasticity of the egg envelope may be less than that of *S. haematocheir*. So egg-to-egg adherence may be observed frequently in other crustaceans.

Figure 9 indicates that envelope plasticity decreases at 40–70 min after egg-laying. Up to 40 min after egg-laying, the attachment point of an egg to the bottom of a dish (*a* in Fig. 1D) never moved when the eggs were rolled with a fine forceps. So changes of the plasticity between 10 and 40 min after egg-laying could be measured exactly. For envelopes 40–70 min after egg-laying, however, the attachment point (*a* in Fig. 1E) moved when the eggs were pushed with forceps. This would be due to a decrease of adhesiveness and an increase in the tensile strength of the envelope. Almost all of the eggs were attached to the ovigerous hairs by 80 min (Fig. 1F). From 80–110 min after egg-laying, the egg envelope could easily be fixed by one forceps and elongated by another forceps. So changes of the plasticity at this time could also be measured exactly. Thus, measurement of the envelope plasticity under the conditions where the attachment point was mobile (40–70 min after egg-laying) may not have been correctly estimated. We speculate that the plasticity at this time might increase linearly between 40 min and 90 min after egg-laying.

A hypothesis concerning the formation of the egg attachment system and ovigerous hair recycling

Our TEM examination of mature ovigerous coats shows electron-dense projections from sublayer E1a of the investment coat that extend to the surface of the hair at intervals of 130–160 nm (Fig. 4). These projections are closely apposed to corresponding periodic, ridge-like structures on the surface of the ovigerous hairs. The ridge-like structures are present on the hairs even when the hairs are not occupied by the investment coat (Fig. 5B). Also, if ovigerous hairs are treated with OHSS, and the investment coat slips off the hair, the ridge-like structures are still present (Fig. 11). Therefore, these ridge-like structures on the ovigerous hairs must be attachment sites for the projections from the investment coat. We propose that these evenly-spaced, linear structures are arranged around the circumference of the hair as shown schematically in Figure 12A. Newly extruded eggs always attach to the hairs at the surface of E1a (Fig. 12B).

Ovigerous hair recycling is summarized in Figure 12C ([1]–[7]); the hairs and coats are shown in cross section. Within 5 min after egg-laying, the egg envelope becomes adhesive (Table 1), and the eggs attach to the cleaned ovigerous hairs [1]. The outer layer of the egg envelope (E1a) develops ridges (small arrows) that project toward attachment sites on layer α1 on the surface of the hair [2]; and the E1a ridges bind to the attachment sites when the egg envelope becomes adhesive (5–40 min after egg-laying; Table 1). The plasticity of the egg envelope (E1) increases greatly 10–30 min after egg-laying, when the investment coat is formed through the action of the female's pleopods [3]. The adhesiveness of the egg envelope begins to decrease 40–50 min after egg-laying (Fig. 9), but envelope plasticity is still maintained for more than 1 h (60–150 h after egg-laying). The funiculus is formed during the first half of this period (*i.e.*, 60–90 min after egg-laying). After the funiculus forms, the female incubates the embryos for nearly 1 month. When embryonic development is complete, the embryos hatch. OHSS (ovigerous-hair stripping substance) in the hatch water (see Saigusa, 1995) attacks the binding of E1a to the attachment sites on the ovigerous hair [4]. As the investment coat dissociates from the hair, the ridges on layer E1a retract (Fig. 11B, C) [5], and the ovigerous hair slips out of the layer E1a [6]. As this process ends, the female picks the remnants of the embryo attachment system off her hairs (see Saigusa, 1982), and these unoccupied hairs are now ready for the next batch of eggs [7].

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